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14. ABSTRACT

The goal of this project is to demonstrate that enzymatically active PSA in the prostatic microenvironment can be used to locally activate either prodrugs or imaging systems. The substrate chosen was a 3 component system composed of a peptide sequence with affinity for PSA, an imaging agent / cytotoxic drug, and a deactivating bridge-linker, which electronically incapacitates the imaging agent /cytotoxin. On PSA mediated release, the peptide sequence is designed to uncouple from the bridge, which then undergoes spontaneous decomposition, releasing imaging agent /free cytoxoxin. The linker selected was 4-amino benzyl alcohol and proof of principle studies were conducted with a tyrosine derivative to which was coupled a series of three image contrast agents. Enzymatic release of imaging agents was achieved on exposure to PSA, however the conjugates were also substrates for the enzyme α-chymotrypsin, limiting their usefulness for screening prostate cancer cells. Specificity for PSA was eventually achieved by conversion to a hexapeptide derivative which underwent selective activation by PSA, releasing imaging agent on exposure to enzyme or prostate cancer tissue. Though specificity for PSA has now been demonstrated, low enzymatic activity of PSA may hinder *in vivo* imaging applications, and a more rapidly degradable substrate is being designed.

15. SUBJECT TERMS
Chemical synthesis, prodrugs, targeted chemotherapy, imaging agents, prostate specific antigen

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Introduction:

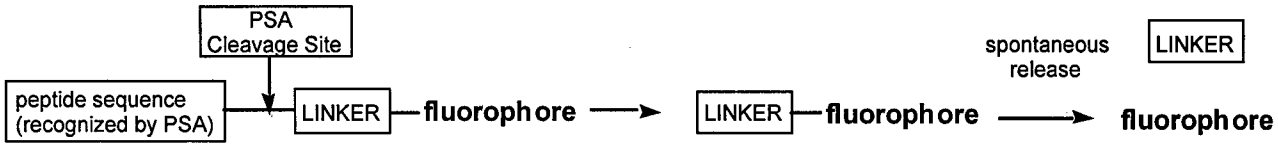
The goal of this project is to demonstrate that enzymatically active PSA in the prostatic microenvironment can be used to locally activate either prodrugs or imaging systems. The research is based on the finding that PSA is enzymatically active, and has a restricted pattern of peptide bond cleavage. The substrates for PSA that we investigated in this project were 3 component systems composed of a peptide sequence with affinity for PSA, an imaging agent and a deactivating bridge, which electronically incapacitates the imaging agent until PSA activates the substrate. Once demonstrated, applications of this work to the *in vivo* imaging of prostatic microenvironments and in the design of cytotoxin prodrugs are anticipated.

Body: As outlined in the Statement of Work, the initial research plan called for design of the three component PSA substrates (objective 1), chemical synthesis of the agents (objective 2), and investigation of enzymatic activation (objective 3). Following completion of this work we anticipated completing required *in vitro* studies (objective 4) before moving to *in vivo* models and subsequent refinement (objectives 5 & 6). As detailed herein, the need to achieve specificity for PSA in the substrates required considerable emphasis on objectives 1-3 during the period of the award, which finally allowed us to progress to objective 4 and beyond during the latter stages.

Objectives 1 &2 : Initial target compound selection and chemical synthesis of all agents

Despite improvements in local therapy and increased awareness, prostate cancer continues to be second only to lung cancer as a cause for cancer deaths in men.[1] Prior investigations show that the presence of prostatectomy Gleason grade ≥4 in the radical prostatectomy specimen is the most important predictor of progression following surgery.[2] Unfortunately, the transrectal ultrasound guided sextant sampling of the prostate is subject to sampling error, and therefore biopsy Gleason grade will underestimate prostatectomy Gleason grade 4 or 5 disease in as many as 40% of men with clinically localized disease.[3] Therefore, an imaging method capable of identifying Gleason grade ≥4 disease within the prostate gland could provide the basis for patient selection for more aggressive initial therapeutic approaches.[4] A number of image contrast enhancing agents have been studied for use in conjunction with ultrasound methods of detection.[5] However, immunohistochemical studies have also shown that Gleason grade bears an inverse correlation with the concentration of enzymatically active prostate specific antigen (PSA).[6] PSA is a serine protease; however, PSA in serum (but not in the prostatic microenvironment) is rapidly inactivated by binding to serum proteins.[7] An attractive possibility, therefore, would be the design of an imaging system, which exploits the enzymatic efficiency of PSA in the prostatic microenvironment. Our strategy was to conjugate a proteinogenic PSA

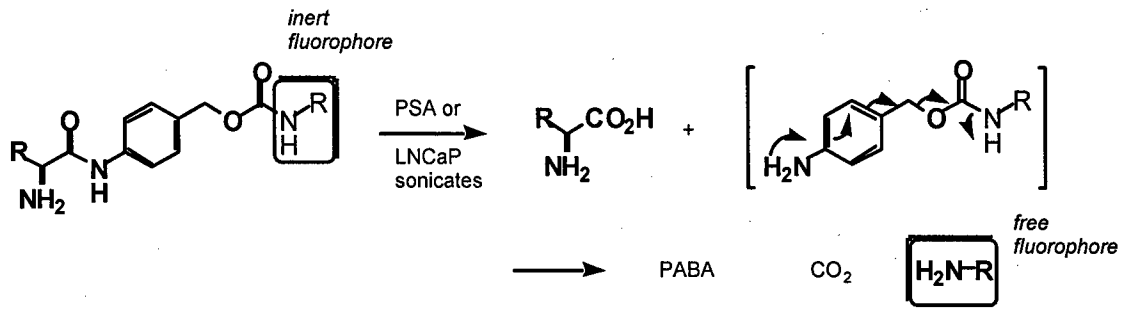
substrate to a masked fluorophore via an inert spacer/linker group, such that the free fluorescent molecule is liberated on proteolysis (Scheme 1).[8]



Scheme 1. Three component system for PSA activated image contrast agent

Choice of linker

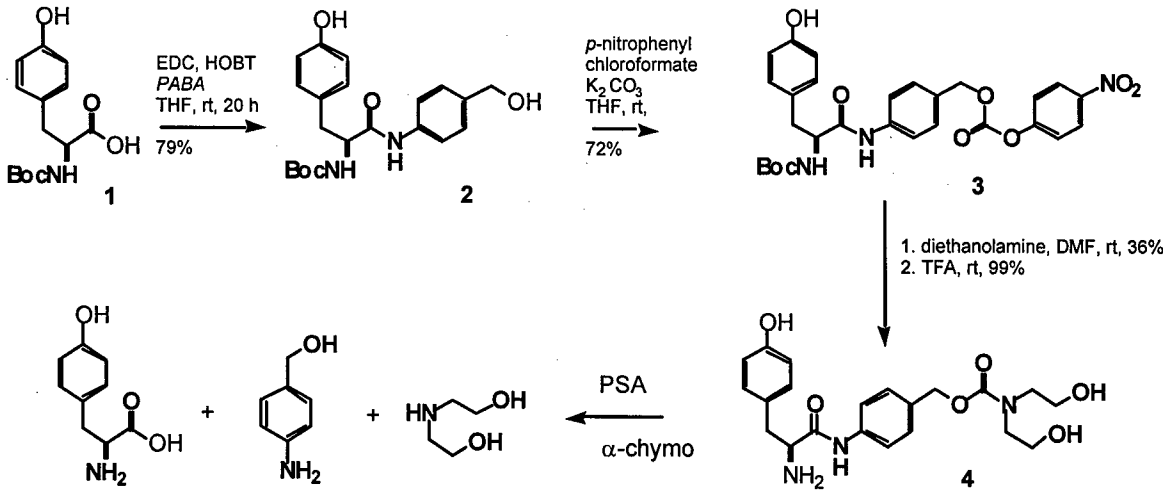
Our preferred choice for the inert linker was the *p*-aminobenzyl alcohol pioneered by Katzenellenbogen.[9] This allows coupling of peptide based enzyme substrates through the N terminus, with the alcohol group incorporated into a carbamate which masks the amino containing molecule targeted for delivery. Selective enzymatic hydrolysis of the amide group results in (a) generation an exomethylene iminium ion which is then capured by water to regenerate the free linker (b) concomitant expulsion of CO_2, rendering the reactions essentially irreversible and (c) expulsion of the free amine as shown in Scheme 2.[9] The key is to harness amino containing substrates in the system where differences in the chemistry between the carbamate and amino form are pronounced, and we have previously employed this method for enzyme mediated cytotoxin release.[10] Anilino containing fluorophores were deemed excellent substrates for this system, as discernable differences in UV and fluorescence characteristics would be expected for the free aniline as opposed to the linked carbamate form.



Scheme 2 . PSA mediated release of Tyr-linker conjugates

Choice of enzyme substrate

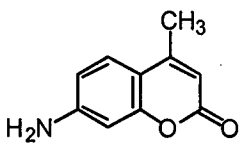
Though a number of high-affinity peptide substrates for PSA have been identified, we initially wished to provide proof-of-principle with a minimal substrate and selected tyrosine conjugates for examination. In addition to precedent for PSA mediated hydrolysis of tyrosyl conjugates, such derivatives would also be substrates of α–chymotrypsin, an important and well studied enzyme.[8] Accordingly, a dummy substrate **4** was prepared to assess proof of principle for the release methodology, and to perfect coupling chemistry (Scheme 3). Boc tyrosine was converted to amide **2** then the carbamate precursor assembled by preparation of the 4-nitrophenyl carbonate **3**. Nucleophilic displacement gave only moderate yields of the Boc carbamate when using close stoichiometry (36% with 4:1 ratio), although near quantitative yields could be obtained when using large excesses of amine (>10 eq.). With the substrate in hand, enzymatic release was tracked using both α-chymotrypsin and PSA. Substantial release was observed within 12h in both cases (Table 1) and despite numerous attempts only tyrosine, 4-aminobenzyl alcohol and diethanolamine were detected, suggesting the intermediate anilino carbamate has a short half-life.



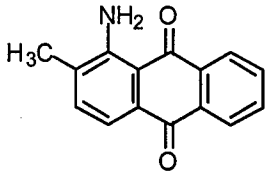
Scheme 3. Proof of principle: Enzymatic release of amine from a *p*-aminobenzyl carbamate-amino acid conjugate

Choice of fluorophores

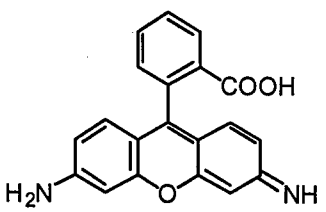
With proof of concept for release of amino containing prodrugs established, we turned attention to selection of appropriate fluorophores. For our initial studies three readily available fluorophore dyes were selected -



7-AMC

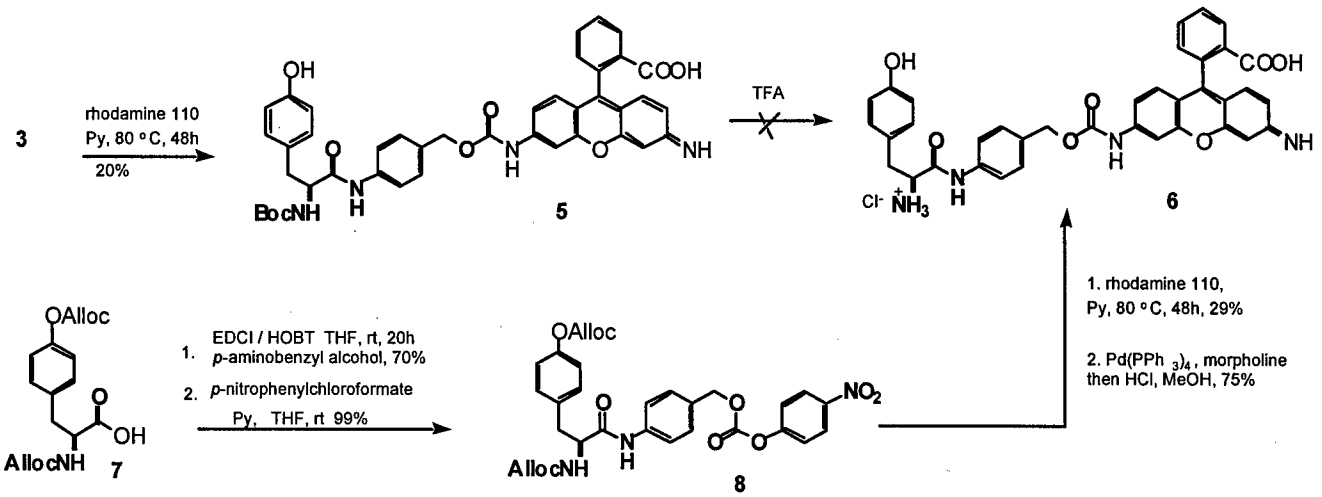


disperse orange 11



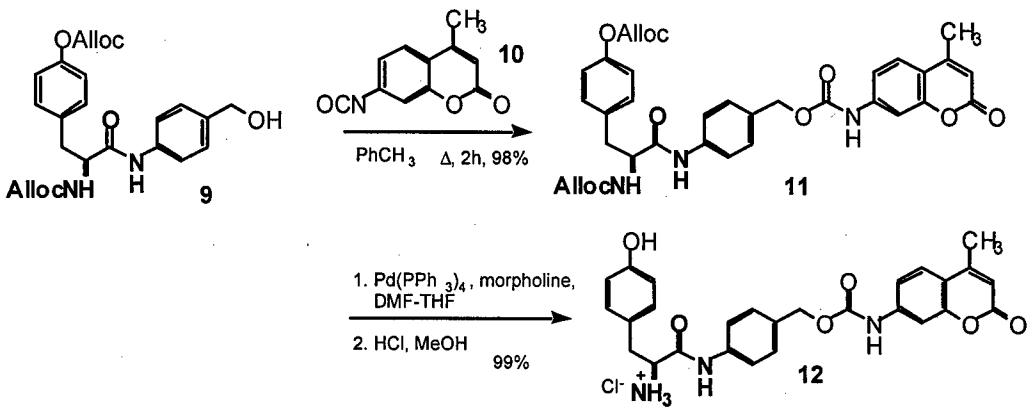
Rhodamine 110

aminomethyl coumarin (7-AMC), disperse orange 11, and rhodamine 110 on the basis that their UV characteristics are all greatly influenced by the electron donating capacity of the anilino nitrogen group. Commencing with the previously available carbonate **3**, coupling with free rhodamine gave **5** cleanly, albeit in low yield (Scheme 4). However, all attempts to unmask the Boc group resulted in decomposition of the molecule, rendering target **6** unisolable. Remedy was found using the alternate bis-alloc substrate **8**, which was prepared from commercially available building block **7** under analogous conditions. Nucleophilic displacement followed by unmasking using Pd chemistry,[11] allowed isolation of the hydrochloride salt **6** in good yield, and the product was freely soluble in assay buffer media.



Scheme 4. Preparation of rhodamine conjugate via alloc protected building block

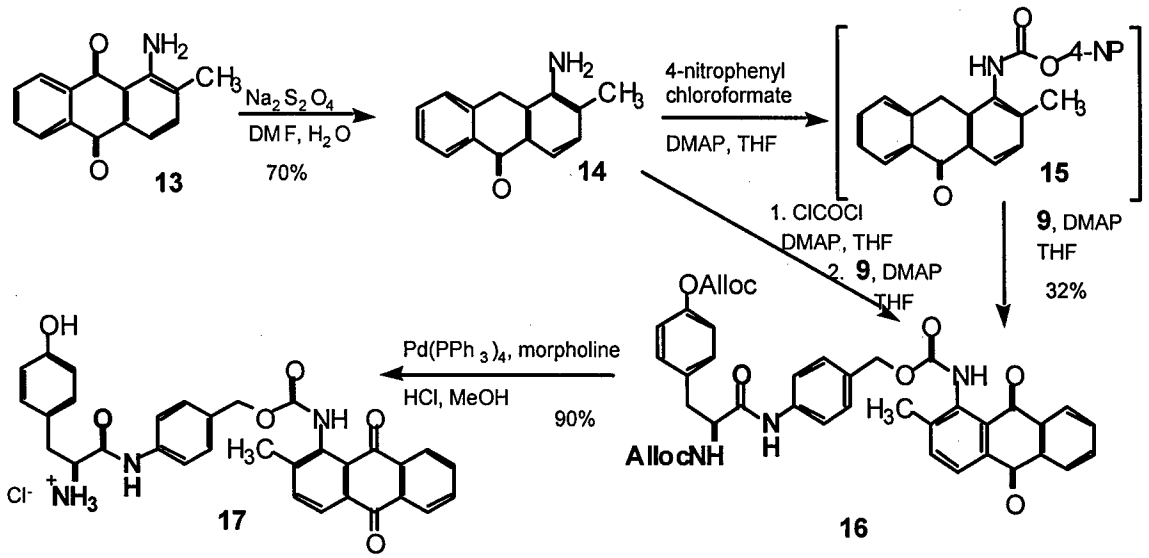
With the alloc route in hand, the coumarin analog **12** was next prepared. This involved coupling of the *p*-aminobeznyl amide prepared in Scheme 4 (**9**) with isocyanate **10** to give the masked analog **11** (Scheme 5). The alloc derivative underwent similarly clean deprotection to give **12** on workup, the product also soluble in buffer media. Finally, hoping to exploit the benefits of intramolecular hydrogen bonding, the



Scheme 5. Preparation of tyrosyl aminomethylcoumarin conjugate

anthraquinone conjugate **17** was assembled (Scheme 6). This necessitated selective removal of the quinone

carbonyl group of aminomethylanthraquinone **13** with dithionite to form required carbamoyl building block **14**. Conversion to the intermediate *p*-nitrophenyl carbamate **15** was inefficient, giving a complex mixture which allowed only low recovered yields of the subsequently coupled product **16**. However, reaction with phosgene followed by coupling with **9** gave alloc derivative **16** directly, reoxidation taking place during workup. This compares favorably with the corresponding conversion via **15**, with yields of up to 80% attainable on scale-up. Finally, unmasking allowed isolation of the hydrochloride salt of anthraquinone substrate **17** in good yield.



Scheme 6. Preparation of tyrosyl aminomethylanthraquinone conjugate

Objective 3 Enzymatic activation studies

With three substrates in hand, spectroscopic and enzymatic studies were conducted to establish proof of concept for use as image contrast agents. Enzymatic release of the fluorophores was probed using fresh, enzymatically active PSA and chymotrypsin, using UV detection to quantitate (and fluorescence in the case of **12**). Release of fluorophore correlated with release of *p*-aminobenzyl alcohol and tyrosine, confirming the function of the self-immolative linker. As can be seen (Table 1), though proof-of-concept is established, in the present examples, α-chymotrypsin is more effective than PSA at cleavage. Though this is unsurprising, more complex oligopeptide substrates are known whose specificity for PSA outranks chymotrypsin significantly, and it was decided to elaborate the substrate with the most selectivity and activity (**12**) to incorporate these recognition elements.

Table 1 Enzyme mediated release of chromophores[#]

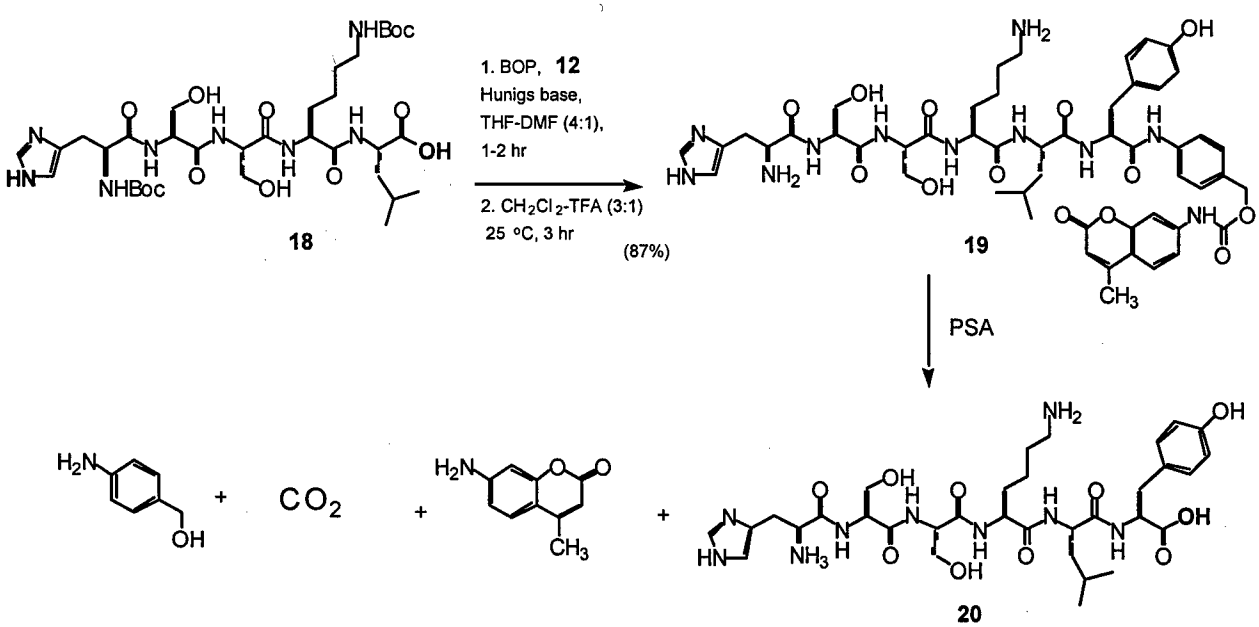
Entry	Substrate	UV λ_{max} conjugate	UV λ_{max} free	α-chymotrypsin[a]	PSA[a]
1	**4**	n/a	n/a	15	5
2	**6**	300	497	9	4
3	**12**	328[b]	352[c]	24	10
4	**17**	285	486	18	6

- mM/h/mg fluorophore released; b fluorescence emission λ_{max} 397; c. fluorescence emission λ_{max} 435

[#]Substrates and controls were incubated for 24-120 h at 37°C. Specific activity was determined on the basis of mM released fluorophore per unit time per unit mass of enzyme.

Objective 6: optimization of lead compound

Based on the work of Denmeade the HSSKL motif was selected and a synthetic route to a chimera of **12** investigated. After numerous iterations, the most successful (and economical) route was to couple tris-Boc protected HSSKL (**18**) with **12** via the BOP route, which, following deprotection gave hexapeptide substrate **19** in good yield (Scheme 7). Enzymatic studies confirmed the hypothesis, with the substrate completely selective for PSA (Table 2). *This is a highly significant finding and a key discovery in that it now sets the stage for production of large numbers of chemical libraries based on the linker strategy.* Though overall less active than **12**, the selectivity demonstrated by **19** is noteworthy, and is now the basis for ongoing development of appropriate *in vitro* and *in vivo* studies. Several improvements to the basic system can be envisioned, tailored to the desired application, based on the synthetic chemistry platform outlined. For example, linker architecture has been shown to have a marked impact on substrate half-life in three component systems,[9,11] suggesting that enhanced activity may be attainable, e.g. with halogen containing



Scheme 7. Preparation & activation of HSSKLY aminomethylcoumarin conjugates

linker moieties (vide infra).

Table 2 Enzyme mediated release of optimized derivatives[#]

Entry	Substrate	UV λ_{max} conjugate	UV λ_{max} free	α-chymotrypsin[a]	PSA[a]
1	**12**	328[b]	352[c]	24	10
2	**19**	328[b]	352[c]	0	4.8

- mM/h/mg fluorophore released; b fluorescence emission λ_{max} 397; c. fluorescence emission λ_{max} 435

[#]Substrates and controls were incubated for 24-120 h at 37°C. Specific activity was determined on the basis of mM released fluorophore per unit time per unit mass of enzyme.

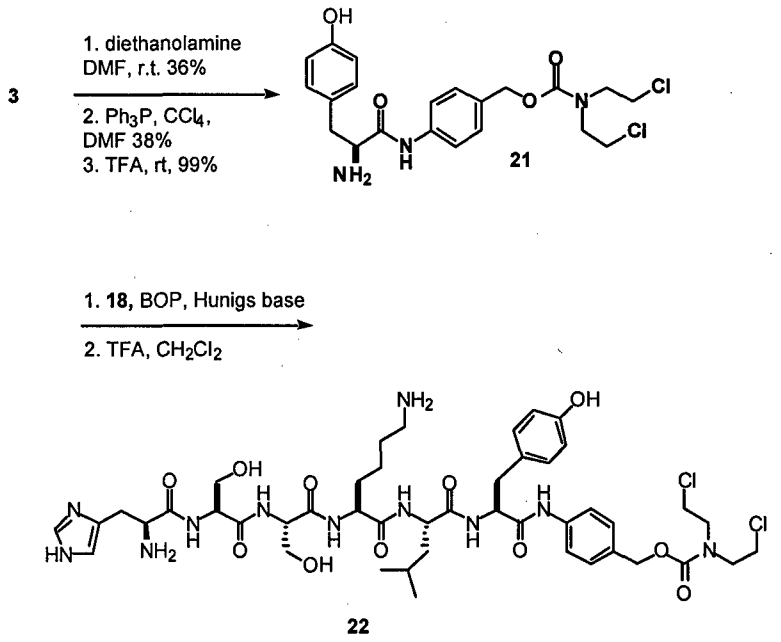
For application with *in vivo* analysis it will be necessary to employ fluorophores with spectral characteristics tailored to match imaging devices. Contrast agents in the near IR range (e.g. the Cy dye family) may prove desirable,[12] in that the conjugated amino function has a profound influence on its quantum yield.[13] The coupling chemistries described herein for amino substituted fluorophores offer flexibility towards this goal, providing the potential for *in situ* CCD based near-IR imaging of systemic agents that are locally activated under *in vivo* conditions.[12] Besides image contrast agents, the system may also prove promising for the slow release of prostate specific chemotherapeutics, where a systemic drug conjugate could be degraded by PSA in the prostatic microenvironment (vide infra).[14]

Objective 4 in vitro analysis

With the PSA selective candidate compound in hand, we have now initiated imaging in live PC cells using the Keck 3D confocal microscopy suite. In addition to LnCaP cells, as controls we are using PSA negative PC-3 cells and also Cos cells. Substrate **19** is visible using 2 laser photonics and is distinctly different from free 7-AMC dye allowing us to track enzymatic release of fluorophore conveniently. These studies are ongoing at the time of writing the report and will be published / disseminated in due course.

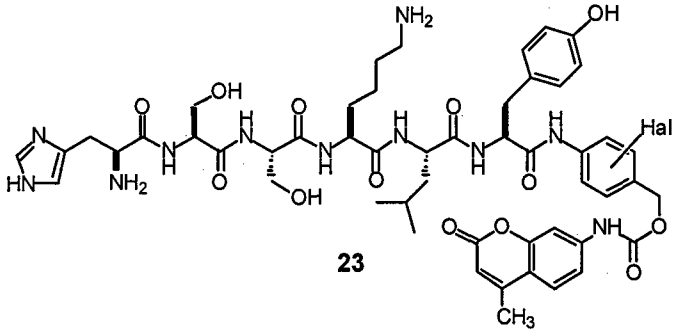
Current / Future objectives

Based on success preparing HSSKL linker conjugates and their selectivity of activation by PSA, we are now investigating the application of this core technology in PSA activated cytotoxic prodrugs. The first candidate of this series is mustard agent conjugate **22**, which can be prepared from **18** via **21** according to the procedure in Scheme 8. We will investigate this compound in a series of *in vitro* cytotoxicity studies, and if successful we will then initiate *in vitro* tumor xenograft studies to determine efficacy.



Scheme 8. Preparation of HSSKLY -linker alkylating agent prodrug

Another line of current investigation is to prepare more activated linker derivatives in the hope that improved leaving group ability in the [reversible] peptide bond cleavage will translate to more efficient PSA mediated activation and release of imaging agents. Specific candidates have been identified (**23**, X=F, Cl) and chemical synthesis is underway. Should these prove successful, Cy dye analogs with activity in the near-IR range will also be pursued.



Key research accomplishments:

- Demonstration that self-immolative linker [4-amino benzyl alcohol] can be used to release and activate image contrast agents from enzyme substrates
- Confirmation that PSA can trigger simple tyrosyl linked substrates to release small molecules
- Efficient chemical synthesis of a family of PSA activated image contrast agents
- Successful synthesis of a substrate with specificity for activation by PSA

- Development of a flexible chemical synthesis procedure to allow preparation of libraries of PSA activated toxins and imaging agents

Reportable outcomes:

Publications already in print

A Prospective, Multicenter, Randomized Phase II Trial of the Herbal Supplement, PC-SPES, and Diethylstilbestrol in Patients with Androgen-Independent Prostate Cancer, William K. Oh, Philip W. Kantoff, Vivian Weinberg, Graham Jones, Brian I. Rini, Mika K. Derynck, Robert Bok, Matthew R. Smith, Glenn J. Bubley, Robert T. Rosen, Robert S. DiPaola, Eric J. Small, *J. Clin. Oncol.* **2004**, *22*, 3705.

Image contrast agents activated by prostate specific antigen (PSA), Graham B. Jones, Longfei Xie, Ahmed El-Shafey, Curtis F. Crasto, Glenn J. Bubley and Anthony V. D'Amico, *Bioorganic and Medicinal Chemistry Letters* **2004**, *14,* 3081

Presentations

PSA Activated prodrugs as prostatic imaging agents , 227th ACS National Meeting, Anaheim, CA, March 28-April 1, 2004

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NIH SPORE award $1.6 million [Bubley, co-PI]
NIH SPORE subcontract $50,000 [to Jones]

Trainees Mentored

Ahmed El-Shafey [Staff scientist - bioanalytical division, Pacific Northwestern Laboratories]
Ajay Purohit [BMS molecular imaging division]
Curtis Crasto [Assistant Professor,University of New Hampshire]
Duckhee Lee [Research Scientist, Korea National University]
Walter Samaniego [Senior Chemist, Florida]
James Weinberg [Staff scientist, Northeastern University]
Jude Mathews [Epix pharmaceuticals]
Longfei Xie [Pfizer Research]
Jane Li [Current student]
Yiqing Lin [Current student]

Degrees Supported

Longfei Xie MS 2004
Ahmed El-Shafey PhD 2003
Ajay Purohit PhD 2002
Jane Li MS 2005

Promotions:

Graham Jones – Appointed Chair of Department of Chemistry & Chemical Biology

Conclusions:

A three component system comprised of enzyme substrate, inert linker and fluorophore has been designed and activation by chymotrypsin and PSA demonstrated. A hexapeptide chimera of one of these is selectively activated by PSA, and the results support application in the *in vitro* and *in vivo* evaluation of more complex

functional substrates, for use as image contrast agents and chemotherapeutics. The use of this technology for the development of prostate cancer therapies and imaging systems is an attractive possibility, though for optimum deployment more active substrates may be desirable. One possibility is to enhance the leaving group ability of the linker entity, possibly through introduction of halogen atoms to the aryl ring. These possibilities are being investigated in the PI's laboratory at the time of writing.

Medical applications of work are a distinct possibility. It is expected that the findings uncovered herein will lead to the design of molecular therapeutics and imaging agents capable of selective activation in prostate cancer tissue. Such agents typically require several years of clinical trials for optimization, but the basic research uncovered will assist in reducing the development time.

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Appendices:

1. Experimental procedures for chemical synthesis and enzymatic assay of agents.

2. Reprint of: Image contrast agents activated by prostate specific antigen (PSA), Graham B. Jones, Longfei Xie, Ahmed El-Shafey, Curtis F. Crasto, Glenn J. Bubley and Anthony V. D'Amico, *Bioorganic and Medicinal Chemistry Letters* 2004, *14,* 3081.

Appendix:

1. Experimental Procedures

HSSKL conjugates and protected Y derivatives were supplied by BACHEM. Unless otherwise stated, all other reagents were purchased from the Aldrich Chemical Company and used as supplied. ^{1}H and ^{13}C NMR spectra were obtained either on a 300MHz Varian Mercury, 300 MHz Bruker AC 300, or 500 MHz Varian Unity machine ($CDCl_3$ unless otherwise stated). Combustion analyses were performed on a Carlo-Erba EA1108 system at the Northeastern University Microanalytical Facility. Mass spectra were conducted at the University of Illinois, Urbana Champaign. Chromatographic separations were made using E Merck 230-400 mesh 60H silica gel, or using a Harrison Research Inc. radial chromatotron unit.

Boc-L-tyrosyl 4-(hydroxymethyl)anilide **(2)**

To a solution of *para*-aminobenzyl alcohol (88 mg, 0.712 mmol) in anhydrous THF (10 ml) cooled to 0 °C was added HOBt (96 mg, 0.712 mmol), boc-tyrosine (200 mg 0.712 mmol), then EDC (150 mg, 0.783 mmol). The mixture was stirred at 0 °C for 2 h and at r. t. for 12 h. The solution was washed with HCl (5%, 2 ml), $NaHCO_3$ (5%, 1 ml), then the organic extracts dried over Na_2SO_4. After condensation *in vacuo* the residue was purified by silical gel chromatography (ethyl acetate: hexanes = 2:1 eluent) to give **2**, (216 mg, 78.5%) as a white solid m.p. 142-14 °C (dec.); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.45 (d, *J*=8.1 Hz, 2H), 7.28 (d, *J*=8.1 Hz, 2H), 7.06 (d, *J*=8.1 Hz, 2H), 6.68 (d, *J*=8.1 Hz, 2H), 4.55 (s, 2H, CH_2), 4.33 (t, 1H,), 2.78-3.40 (m, 2H), 1.40 (s, 9H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 176.1, 175.9, 160.6, 160.0, 141.5, 141.2, 141.1, 134.4, 131.9, 131.5, 124.6, 124.5, 119.3, 83.8, 67.8, 61.2, 41.9, 31.8; MS (ESI) *m/z* 387 $(M+H)^+$; HRMS (ESI) calc. for $C_{21}H_{27}N_2O_5$ $(M+H)^+$ *m/z* 387.1920, found 387.1916.

Boc-L-tyrosyl 4-(4-nitrophenoxycarboxyl)methylanilide **(3)**

To a solution of alcohol **2** (200 mg, 0.5175 mmol) in fresh distilled anhydrous THF (10 ml) were added 4-nitrophenyl chloroformate (110 mg, 0.5434 mmol) and anhydrous pyridine (44 μl, 0.5434 mmol). The mixture was stirred at r.t. for 12h. The resulting precipitate was filtered and the filtrate evaporated. The residue following evaporation was purified by silical gel chromatography (ethyl acetate: hexanes = 1:1 eluent) to give **3**, (204.3 mg, 71.7%) as a white solid m.p. 175-179 °C; ^{1}H-NMR (300 MHz $CDCl_3$) δ 8.31 (d, *J*=8.7 Hz, 2H), 7.53 (d, *J*=8.7 Hz, 2H), 7.45 (d, *J*=8.7 Hz, 2H), 7.39 (d, *J*=8.7 Hz, 2H), 7.07 (d, *J*=8.7 Hz, 2H), 6.69 (d, *J*=8.7 Hz, 2H), 5.24 (s, 2H, CH_2), 4.35 (t, 1H,), 2.8-3.8 (m, 2H), 1.40 (s, 9H); ^{13}C-NMR (375 MHz $CDCl_3$) δ 172.0, 164.0, 156.0, 150.4, 140.6, 138.4, 138.2, 135.2, 131.8, 131.8, 130.4, 130.2, 128.8, 127.7, 125.9, 125.1, 120.8, 120.286, 115.6, 115.3, 115.1, 69.0, 57.0, 54.0, 39.0, 27.5; $C_{28}H_{29}N_3O_9$ calc. C, 60.98; H, 5.30; N, 7.62; found, C, 61.31; H, 5.54; N, 7.26.

L-Tyrosyl 4-(*N,N*-bis(2-hydroxyethyl)carbamoyl)methylanilide **(4)**

A solution of diethanolamine (0.276 g, 2.6 mmol) in anhydrous DMF (5.0 mL) was added to a solution of **3** (0.32 g, 0.58 mmol) in anhydrous DMF (5.0 mL). The mixture was stirred at r.t. for 42 h, then quenched with water (50 mL). The mixture was extracted with ethyl acetate (3 x 30 mL) and the combined extracts washed with brine (1 x 15 ml), then dried (Na_2SO_4). The solution was concentrated *in vacuo* and the residue was purified by flash chromatography (10% methanol in chloroform) to give Boc-L-tyrosyl 4-(*N,N*-bis(2-hydroxyethyl)-carbamoyl)methylanilide (0.163 g, 36%) as a hygroscopic oil. The entire anilide (0.314 mmol) was treated with trifluoroacetic acid (6 mL) under argon, stirred for 12 h and then the TFA was removed by aspiration. The resulting gum was purified by chromatography (1: 9 methanol:chloroform) to give **4**, (166.5 mg, 99%) as a colorless foam; ^{1}H-NMR (300 MHz, d_6-acetone): δ 7.77-6.83 (m, 10H); 5.35 (s, 1H); 4.65 (s, 2H); 3.96 (m, 4H); 3.75 (m, 1H); 3.55 (m, 1H); 3.40 (m, 7H); 3.01 (s, 1H); 2.85 (s, 1H); $C_{21}H_{27}N_3O_6$ calc. C, 60.42; H, 6.52; N, 10.06; found, C, 60.77; H, 6.73; N, 9.71.

Boc Rhodamine conjugate **(5)**

Carbonate **3** (91 mg, 0.1637 mmol) and rhodamine 110 (60 mg 0.1637 mmol) were dissolved in anhydrous pyridine (10 ml) and the solution stirred at 80 °C for 2 days. The solution was condensed *in vacuo* and the residue was was purified by silical gel chromatography (ethyl acetate: hexanes = 3:1 eluent) to give **5**, (23.8 mg, 19.6%) as a yellow oil together with recovered rhodamine (40 mg); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.91 (d, *J*=7.2 Hz, 1H), 7.54-7.68 (m, 5H), 7.496 (s, 1H), 7.40(d, *J*=8.1 Hz, 2H), 7.27 (d, *J*=8.7 Hz, 2H), 7.11 (d, *J*=7.5 Hz, 1H), 6.90-7.04 (m, 4H), 6.63 (d, *J*=8.1 Hz, 2H), 6.56 (d, *J*=9.0 Hz, 2H), 6.48 (d, *J*=2.4 Hz, 2H), 6.42 (d, *J*=8.7 Hz, 1H), 6.33 (dd, *J*=8.7, 2.4 Hz, 1H), 5.06 (s, 2H, CH_2), 4.28 (t, 1H), 2.7-3.0 (m, 2H), 1.31 (s, 9H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 171.4, 170.8, 156, 154.3, 153.2, 152.5, 151, 144.9, 141.7, 138, 135.8, 132.5, 130.4, 129.9, 129, 128.8, 128.5, 127.6, 127.4, 125.0, 124.4, 120.5, 66.5, 56.8, 37.2, 29.5, 28.0; MS (ESI) *m/z* 743 $(M+H)^+$; HRMS (ESI) calcd for $C_{42}H_{38}N_4O_9$ $(M+H)^+$ *m/z* 743.2717, found 743.2746.

(bis)Alloc tyrosyl 4-(hydroxymethyl)anilide **(9)**

To a solution of bis-alloc tyrosine (2.26 g, 6.476 mmol) in anhydrous THF (50 ml) was added 4-aminobenzylalcohol (800 mg, 6.476 mmol), EDCI (1.5 g, 7.77 mmol), then HOBT (874 mg, 6.476 mmol). The mixture was stirred at r.t. for 12 h, then condensed *in vacuo*. The residue was dissolved in ethyl acetate (30 ml) and the solution washed with HCl (5%, 2 ml), $NaHCO_3$ (5%, 1 ml), then the organic extracts dried over Na_2SO_4. After condensation *in vacuo* the residue was purified by silical gel chromatography (ethyl acetate: hexanes = 2:1 eluent) to give the title compound (2.09g, 71%) as a pale solid m.p. 177-119 °C; ^{1}H-

NMR (300 MHz, $CDCl_3$) δ 7.45 (d, *J*=8.7 Hz, 2H), 7.27-7.32 (m, 4H), 7.08 (d, *J*=8.7 Hz, 2H), 5.80-6.10 (m, 2H), 5.13-5.42(m, 4H), 4.70(dd, 1H, *J*=5.7, 1.5Hz), 4.55 (s, 2H, CH_2), 4.49 (t, 1H), 2.90-3.20 (m, 2H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 172.0, 157.0, 154.0, 150.0, 138.0, 137.0, 135.0, 133.0, 132.0, 130.3, 127.4, 121.0, 120.0, 118.0, 117.0, 69.0, 65.0, 63.0, 57.0, 38.0; MS (ESI) *m/z* 455 $(M+H)^+$; HRMS (ESI) calcd for $C_{24}H_{27}N_2O_7$ $(M+H)^+$ *m/z* 455.1818, found 455.1827.

Bis (alloc)-L-tyrosyl 4-(4-nitrophenoxycarboxyl)methylanilide (**8**)

To a solution of **9** (100mg, 0.22 mmol) THF (5 ml) was added 4-nitrophenyl chloroformate (57 mg, 0.28 mmol) and anhydrous pyridine (23 µl, 0.28 mmol). The mixture was stirred at r.t. for 12 h. then the formed precipitate filtered and the filtrate recovered and evaporated.. This residue was purified by silical gel chromatography (ethyl acetate: hexanes = 1:2 eluent) to give **8**, (143.3 mg, 98.8%) as a white solid m.p. 87-90 °C (dec.);. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.25 (d, *J*=9.0 Hz, 2H), 7.30-7.44 (m, 6H), 7.23 (d, *J*=9.0 Hz, 2H), 7.10 (d, *J*=8.7 Hz, 2H), 5.78-6.09 (m, 2H), 5.65 (d, *J*=5.7 Hz, 1H), 5.10-5.50(m, 5H), 4.72 (d, *J*=5.7 Hz, 2H), 4.53-4.60 (m, 3H), 3.14(s, 1H), 3.11(s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 170.2, 156.1, 155.8, 155.1, 153.8, 152.7, 150.5, 145.8, 137.9, 134.3, 132.4, 131.2, 130.7, 130.6, 129.9, 126.9, 126.4, 125.5, 122.5, 122.0, 121.7, 120.7, 119.8, 118.5, 116.1, 70.9, 69.5, 66.5, 55.5, 37.5; MS (ESI) *m/z* 620 $(M+H)^+$; HRMS (ESI) calcd for $C_{31}H_{30}N_3O_{11}$ $(M+H)^+$ *m/z* 620.1880, found 620.1892.

Bis(alloc) L-tyrosyl rhodamine

Carbonate **8** (69 mg, 0.112 mmol) and rhodamine 110 (40 mg 0.112 mmol) were dissolved in anhydrous pyridine (10 ml) the solution stirred at 80°C for 2 days. The solution was evaporated to dryness and the residue was purified by silical gel chromatography (ethyl acetate: hexanes = 2:1 eluent) to give the title compound (26 mg, 28.6%) as a yellow oil together with recovered rhodamine (16 mg); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.34 (s, 1H), 7.98 (d, *J*=7.2 Hz, 2H), 7.50-7.70 (m, 5H), 7.00-7.40 (m, 8H), 6.68(d, *J*=8.4 Hz, 1H), 6.64 (d, *J*=8.7 Hz, 1H), 6.49 (d, *J*=8.4 Hz, 1H), 6.43 (s, 1H), 6.20 (d, *J*=8.1 Hz, 1H), 5.70-6.12 (m, 2H), 5.12-5.50(m, 4H), 5.06 (s, 2H, CH_2), 4.72 (dd, *J*=5.7, 0.9 Hz, 2H), 4.50-4.70 (m, 3H), 3.11 (s, 2H); ^{13}C-NMR (75 Mhz, $CDCl_3$) δ 172.5, 170.4, 156.8, 155.8, 154.0, 153.8, 152.8, 152.2, 150.8, 150.2, 149.2, 140.5, 137.5, 135.5, 134.5, 132.8, 132.2, 131.3, 130.6, 129.9, 129.2, 128.9, 127.8, 127.0, 125.2, 124.2, 121.5, 120.5, 119.8, 118.2, 115.8, 114.5, 113.8, 111.9, 108.2 106.5, 101.8, 69.2, 66.8, 66.2, 56.5, 38.2; MS (ESI) *m/z* 811 $(M+H)^+$, HRMS (ESI) calcd for $C_{45}H_{39}N_4O_{11}$ $(M+H)^+$ *m/z* 811.2615, found 811.2606.

L-tyrosyl rhodamine **(6)**

A catalytic amount of $Pd(PPh_3)_4$ (~ 1 mg) and morpholine (0. 5 ml) was added to a degassed solution of bis(alloc) L-tyrosyl rhodamine (14.7 mg, 0.0182 mmol) in anhydrous THF (5 ml). The solution was stirred under argon for 48 h, then the resulting red precipitate was collected by centrifugation, washed with ethyl acetate (2 x 5 ml). The solid was then exposed to a solution of hydrochloric acid (0.5M, 2 ml) in ethyl acetate (5 ml) followed by methanol (1 ml). The resulting (yellow) solution was condensed *in vacuo* solution and the residue was purified by silica gel chromatography (ethyl acetate: methanol = 10:1 eluent) to give **6**, (8.7 mg, 74.7%) as a yellow solid m.p. 213-216 °C; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.38 (d, *J*=5.1 Hz, 1H), 8.23 (s, 1H), 7.80-7.92 (m, 1H), 7.50-7.70 (m, 4H), 7.30-7.48 (m, 2H), 7.20-7.26 (m, 2H), 7.15 (d, *J*=5.1 Hz, 1H), 6.90-7.10 (m, 2H), 6.75 (d, *J*=5.7 Hz, 1H), 6.66 (t, *J*=5.1 Hz, 1H), 5.22 (s, 2H, CH_2), 4.23(t, 1H), 2.84-3.28 (m, 2H); ^{13}C-NMR (75 MHz, CD_3OD) δ 176.2, 171.5, 161.0, 142.0, 141.8, 137.0, 136.7, 136.5, 135.9, 135.8, 135.6, 135.5, 135.4, 134.9, 134.5, 134.3, 134.2, 132.9, 132.8, 131.4, 128.8, 124.9, 121.4, 121.1, 120.9, 119.5, 108.2, 70.8, 61.1, 40.7; MS (ESI) *m/z* 643 $(M+H-2HCl)^+$, HRMS (ESI) calcd for $C_{37}H_{31}N_4O_7$ $(M+H-2HCl)^+$ *m/z* 643.2193, found 643.2215.

(bis)Alloc tyrosyl 4-(hydroxymethyl)anilide coumarin conjugate **(11)**

A solution of (bis)Alloc tyrosyl 4-(hydroxymethyl)anilide (**9**, 0.100g, 0.22 mmol) and coumarin isocyanate (**10**, 66 mg, 0.33 mmol) in toluene (10 ml) were refluxed for 2 h. The solution was condensed *in vacuo* and the residue crystallized from CH_2Cl_2:MeOH:Et_2O to give **11** (138 mg, 98%) as a white solid m.p. 139-142 °C; ^{1}H-NMR ^{1}H NMR (CD_3OD, 500 MHz) δ 7.45 (d, 2H, J=10Hz), 7.41-7.36 (m, 3H), 7.28-7.24 (m, 3H), 7.18-7.14 (m, 3H), 7.02-6.98 (m, 3H), 6.09 (d, 1H, J=1.5 Hz), 5.94-5.86 (m, 1H), 5.82-5.74 (m, 1H), 5.36-5.31 (m, 1H), 5.26-5.23 (m, 1H), 5.18 (d, 1H, J=17.5 Hz), 5.12-5.07 (m, 3H), 4.65-4.63 (m, 2H), 4.45-4.44 (m, 2H), 4.39 (t, 1H, J=7 Hz), 3.53 (br, s, 3H), 3.04 (dd, 1H, J=13.7, 7 Hz), 2.96 (dd, 1H, J=13.7, 7 Hz); MS (ESI) *m/z* 656 $(M+H)^+$;

L tyrosyl 4-(hydroxymethyl)anilide coumarin conjugate **(12)**

A solution of **11** (50 mg, 76.5 mmol) in DMF-THF (1:1, 4 ml) was treated with morpholine (1 ml) and $Pd(Ph_3)_4$ (10 mg). The mixture was stirred at r.t. for 1 h then concentrated in vacuo. The residue was purified by silica gel chromatography (CH_2Cl_2: MeOH: Et_3N: 93:5:2 eluent) to give the title compound (0.041g, 99%) as a white solid m.p. 183-185 °C;.^{1}H NMR (CD_3OD, 500 MHz) δ 7.57 (s, 1H, Ar), 7.58-7.50 (m, 3H, Ar), 7.39-7.35 (m, 3H, Ar), 7.03 (d, *J*= 8.0 Hz, 2H, Ar), 6.72 (d, *J*= 8.0 Hz, 2H, Ar), 6.15 (br, s, 1H, vinyl-H), 5.14 (s, 2H, benzylic), 4.69 (br, s, 5H, *exch.*), 3.60 (dd, *J*= 5.5, 8.0 Hz, 1H, C*H*NH), 3.02 (dd, *J*= 5.5, 14.0 Hz, 1H, CH), 2.75 (dd, *J*= 5.5, 14.0 Hz, 1H, CH), 2.42 (s, 3H, CH_3); ^{13}C NMR (CD_3OD, 75 MHz) δ 169.98, 156.10,

153.86, 153.49, 153.38, 149.64, 142.43, 137.27, 134.17, 132.04, 131.80, 130.63, 130.03, 128.50, 124.93, 120.66, 119.99, 118.89, 117.15, 114.65, 114.52, 111.74, 105.29, 68.82, 56.25, 37.57, 17.93; HRMS calc. for $C_{27}H_{26}N_3O_6$(MH+) *m/z* 488.1822, found 488.1825

De-oxy disperse organge **14**

Sodium dithionite (14 g, 80.41 mmol) was added to a suspension of the disperse orange 11 (**13**, 2 g, 8.439 mmol) in DMF (100 ml) and water (100 ml) and the mixture was heated to 90 °C over 30 min. The mixture was stirred for 48 h. then cooled to r.t. The resulting yellow precipitate was filtered and then purified by silical gel chromatography (hexanes: ethyl acetate =1:1 eluent) to give **14** (1.08 g, 58%) as a yellow solid m.p. 103-105 °C; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.30(dd, 1H, *J*=8.4Hz, 0.9Hz), 7.78(d, 1H, *J*=7.5Hz), 7.54-7.59 (m, 1H), 7.45(d, 2H, *J*=6.9Hz), 7.16(d, 1H, *J*=8.1Hz), 3.87(s, 2H), 3.76(s, 2H), 2.22(s, 3H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 184.7, 141.8, 139.8, 132.7, 132.0, 130.7, 129.2, 126.0, 127.6, 127.2, 126.8, 124.4, 117.8, 28.4, 18.3; MS (ESI) *m/z* 224.2 $(M+H)^+$; HRMS (ESI) calcd for $C_{15}H_{16}NO$ $(M+H)^+$ *m/z* 224.1075, found 224.1075.

Disperse orange-4 Bis-alloc-L tyrosyl carbamate (**16**)

Procedure a:

4-Nitrophenyl chloroformate (511 mg, 2.537 mmol) and DMAP (340 mg, 2.537 mmol) were added to a degassed solution of **14** (514.3 mg, 2.300 mmol) in THF (40 ml). The solution was stirred at room temperature for 24 h. then filtered and the filtrate condensed *in vacuo*. This residue was purified via silica gel chromatography (hexanes: ethyl acetate=4:1), to give (64 mg 0.165 mmol) of crude carbonate **15**. This material was immediately dissolved anhydrous THF (20 ml) and **9** (75 mg, 0.165 mmol) and DMAP (22 mg, 0.165 mmol) added. The solution was stirred at room temperature for 24 h. filtered and the filtrate condensed *in vacuo*. This residue was purified via silica gel chromatography (hexanes: ethyl acetate=1:1) to give **16** (24.9 mg, 31.5%) as a yellow oil; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 10.0(s, 1H), 8.20-8.30(m, 2H), 8.10(d, 1H, *J*=7.5Hz), 7.70-7.80(m, 4H), 7.63(d, 1H, *J*=7.5hz), 7.30-7.50(m, 4H), 7.24(d, 1H, *J*=7.2Hz), 7.11(d, 2H, *J*=8.1z), 5.80-6.20(m, 2H), 5.00-5.50(m, 6H), 4.72(d, 2H, *J*=5.7Hz), 4.40-4.60(m, 3H), 3.10-3.20(m, 2H), 2.41(s, 3H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 186.9, 182.7, 169.3, 154.2, 153.7, 150.7, 150.7, 150.5, 142.5, 138.6, 134.5, 134.4, 133.0, 132.7, 132.5, 132.4, 131.3, 130.6, 129.3, 127.6, 127.1, 126.3, 124.8, 124.4, 121.7, 120.4, 119.8, 118.5, 69.4, 67.3, 66.5, 57.2, 38.0, 20.2; MS (MALDI) *m/z* $(M+Na)^+$ 740.84

Procedure B:

A solution of phosgene (20%) in toluene (1.8 ml, 3.60 mmol) and DMAP (482 mg, 3.60 mmol) were added to

a degassed solution of **14** (400 mg, 1.79 mmol) in THF (20 ml). The solution was stirred at r.t. for 24 h. and the excess phosgene removed with an argon sweep. **9** (750 mg, 1.79 mmol) was then added and the solution stirred at r.t. for 24 h. The mixture was filtered and the filtrate condensed *in vacuo*. This residue was purified via silica gel chromatography (hexanes: ethyl acetate=1:1) to give **16** (145 mg, 22%) plus recovered **9** (639 mg) [yield **83%** based on **9**].

L-Tyrosyl-disperse orange carbamate (**17**)

A catalytic amount of $Pd(PPh_3)_4$ and of morpholine (50 µl) were added to a degassed solution of **16** (38 mg, 0.053 mmol) in anhydrous THF (5 ml). The solution was stirred under argon for 48 h. and the resulting red precipitate recovered by centrifugation, and washed with ethyl acetate (10 ml). The solid was dissolved in methanol (1 ml) the solution condensed *in vacuo* then the residue was purified via silica gel chromatography (ethyl acetate: methanol = 10:1 eluent) to give **17** (26.1 mg, 90%) as a red solid m.p. 221-224 °C; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.19-8.30 (m, 2H), 8.10-8.15(m, 1H), 7.82-7.86(m, 2H), 7.64(d, 1H, *J*=8.1Hz), 7.54 (d, 2H, *J*= 8.4Hz), 7.41(d, 2H, *J*=8.1Hz), 7.10-7.18(m, 2H), 6.86-6.92(m, 1H), 6.76(d, 2H, *J*=8.4Hz), 5.18 (s, 2H), 4.20(t, 1H), 3.0-3.4(m, 2H), 2.40(s, 3H); MS (MALDI) *m/z* $(M+Na)^+$ 572.86; $C_{32}H_{27}N_3O_6$ calc. C, 69.93; H, 4.95; N, 7.65; found, C, 70.31; H, 5.06; N, 7.33.

HSSKLY-tyrosyl-coumarin carbamate conjugate (**19**)

Pentapeptide NH_2-HSSKL-CO_2H (10 mg, 17.6 µmol), Boc anhydride (8.86 mg, 40 µmol) and Et_3N (16 µl, 106 µmol) were stirred at 40 °C for 12 h in DMF (2 ml). The solution was concentrated *in vacuo* the residue redissolved in DMF:THF (1:1, 10 ml) then **12** (9 mg, 20.2 µmol) BOP (10 mg, 22 µmol) and Hunigs base (8 µl, 44 µmol). The mixture was stirred at r.t. for 12 h. The solution was concentrated *in vacuo* and the residue dissolved in MeOH (2 ml) and the solution passed through a silica gel plug (6% MeOH, 94% CH_2Cl_2 eluent). Removal of eluents and concentration *in vacuo* gave the Boc protected derivative of **19** (21.8 mg, 87%) as a colorless gum; (ESI MS 1239 = M^+). A portion of this product (14 mg, 11.1 µmol) was treated with a solution of TFA (25% in CH_2Cl_2, 3 ml) and stirred at r.t. for 3h. then condensed *in vacuo*. The residue was dissolved in DMF (1 ml) and the solution passed through a short plug of silica gel. The eluents were condensed *in vacuo* and the residue washed with CH_2Cl_2 (3 x 1 ml) then hexanes (3 x 1 ml) to give **19** (11.4 mg, 99%) as a pale white solid m.p. 168-173 °C (dec.); ESI-MS 1040.14 (M^+).

General procedure for enzymatic assays

In duplicate, PSA (Cortex Biochem), 20 µL, 0.5 mg/mL, pH 7.4 0.01 M phosphate buffer saline [PBS]) or α-chymotrypsin (Sigma, TLCK-treated, 20 µL, 0.5 mg/mL, pH 7.4 0.01 M PBS), 160 µL PBS, and 20 µL substrate (1.0 mg/mL; 1:1, EtOH:H_2O) were incubated for 24 h at 37°C. Duplicate control reactions containing PBS (180 µL) and substrate (20 µL) solutions were incubated under identical conditions. The two cuvettes were placed in Varian (Cary WinUV) spectrophotometer for 24 hours, and kinetics recorded. To determine release of free fluorophore and linker, substrates and controls were also incubated at 37 °C for 24-120 h then reactions terminated by addition of cold tricholoracetic acid (10% v/v). Enzyme was removed by centrifugation (700 xg, 30 min, 4 °C) using Ultrafree-MC tubes (Millipore), and peak areas for control and reaction mixtures determined by HPLC. Conditions: C-18 reverse phase column (15cm); mobile phase A 99% H_2O + 1% TEA; mobile phase B 99% Methanol + 1% TEA; linear gradient 10-80%; flow rate 1 ml/min; monitoring wavelength = 253 nm. Specific activity (mmol/h/mg) was determined as: [conc.] released substrate (mmole/ml) x total vol. of the assay (ml) / time of the reaction (h) x [conc.] enzyme mg/ml x vol. enzyme (ml).

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Image contrast agents activated by prostate specific antigen (PSA)

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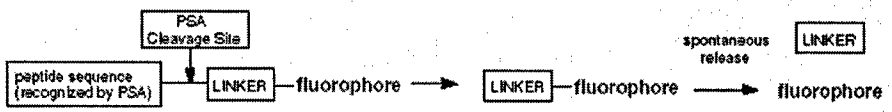
Abstract—A family of image contrast agent conjugates designed to undergo enzymatic activation has been synthesized. The agents underwent activation both with enzymatically active prostate specific antigen and α-chymotrypsin, releasing free fluorophore via cleavage of a three-component system.

Despite improvements in local therapy and increased awareness, prostate cancer continues to be second only to lung cancer as a cause for cancer deaths in men.[1] Prior investigations show that the presence of prostatectomy Gleason grade ≥4 in the radical prostatectomy specimen is the most important predictor of progression following surgery.[2] Unfortunately, the transrectal ultrasound guided sextant sampling of the prostate is subject to sampling error, and therefore biopsy Gleason grade will underestimate prostatectomy Gleason grade 4 or 5 disease in as many as 40% of men with clinically localized disease.[3] Therefore, an imaging method capable of identifying Gleason grade ≥4 disease within the prostate gland could provide the basis for patient selection for more aggressive initial therapeutic approaches.[4] A number of image contrast enhancing agents have been studied for use in conjunction with ultrasound methods of detection.[5] However, immunohistochemical studies have also shown that Gleason grade bears an inverse correlation with the concentration of enzymatically active prostate specific antigen (PSA).[6] PSA is a serine protease; however, PSA in serum (but not in the prostatic microenvironment) is rapidly inactivated by binding to serum proteins.[7] An attractive possibility, therefore, would be the design of an imaging system, which exploits the enzymatic efficiency of PSA in the prostatic microenvironment. Our strategy was to conjugate a proteinogenic PSA substrate to a masked fluorophore via an inert spacer/linker group, such that the free fluorescent molecule is liberated on proteolysis (Scheme 1).[8]

Our preferred choice for the inert linker is the *p*-aminobenzyl alcohol pioneered by Katzenellenbogen,[9] having previously employed this method for enzyme mediated cytotoxin release.[10] Though a number of high-affinity peptide substrates for PSA have been identified, we initially wished to provide proof-of-principle with a minimal substrate and selected tyrosine conjugates for examination of appropriate fluorophores (Scheme 2).[11]



Scheme 1. Three-component system for PSA activated image contrast agent.

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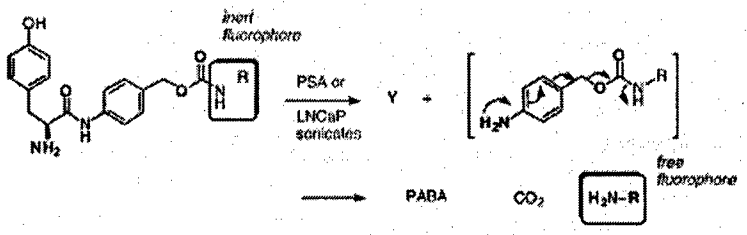
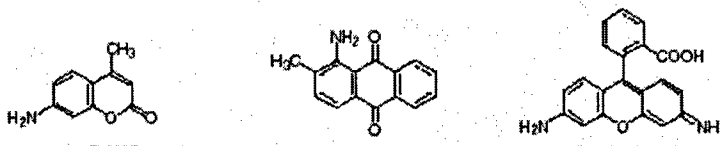


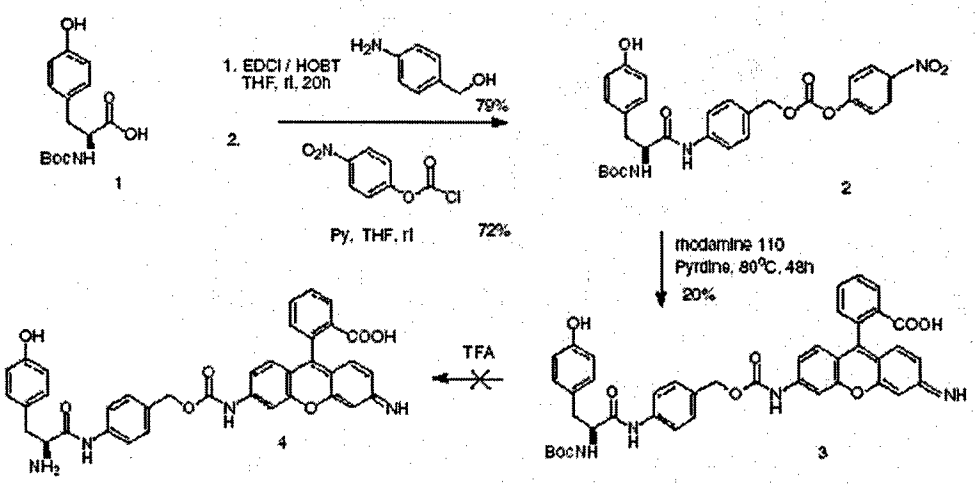
Scheme 2. PSA mediated release of Tyr-linker conjugates.



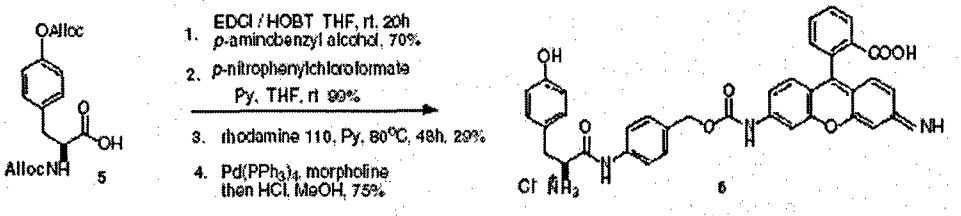
For our initial studies three readily available fluorophore dyes were selected—aminomethyl coumarin (7-AMC), disperse orange 11, and rhodamine 110.

Commencing with commercially available Boc-tyrosine, carbodiimide coupling with *p*-aminobenzyl alcohol, followed by reaction with *p*-nitrophenylchloroformate gave carbonate **2** without incident (Scheme 3). Coupling with free rhodamine gave **3** cleanly, albeit in low yield. However, all attempts to unmask the carbamate group resulted in decomposition of the molecule, rendering **4** unisolable. Remedy was found using the alternate bis-alloc substrate **5**, which under analogous conditions gave the intermediate carbonate, and subsequently underwent rhodamine coupling and unmasking using the Pd route,[12] to allow isolation of the hydrochloride salt **6** in good yield, and which was soluble in assay buffer media (Scheme 4).

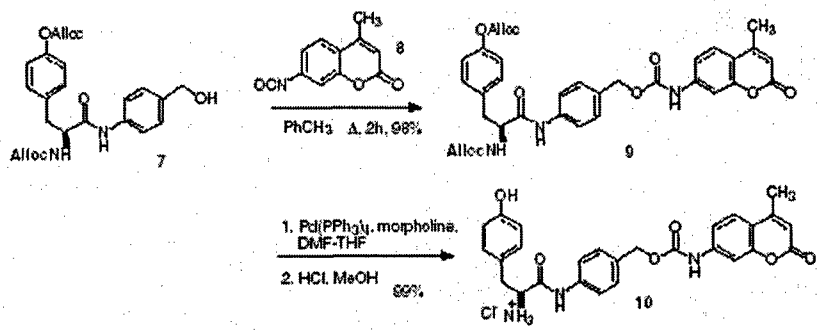
With the alloc route in hand, the coumarin analog **1** was next prepared. This involved coupling of the ca bonate used in Scheme 4 (**7**) with isocyanate **8** to give th masked analog **9**, which underwent clean deprotectio to give **10** on workup (Scheme 5). Finally, hoping t exploit the benefits of intramolecular hydrogen bondin the anthraquinone conjugate **14** was assembled. Th necessitated selective removal of the quinone carbon group of aminomethylanthraquinone to allow formi tion of the required carbamoyl building block **1** (Scheme 6). Conversion to the *p*-nitrophenyl carbama was inefficient, giving a complex mixture, which allowe only low recovered yields of **12**. However, reaction wit phosgene followed by coupling with **7** gave alloc pr tected adduct **13** directly, reoxidation taking place du ing workup. This compares favorably with th corresponding conversion of **12** to **13** and proved rel able on scale-up. Finally, unmasking allowed isolatio



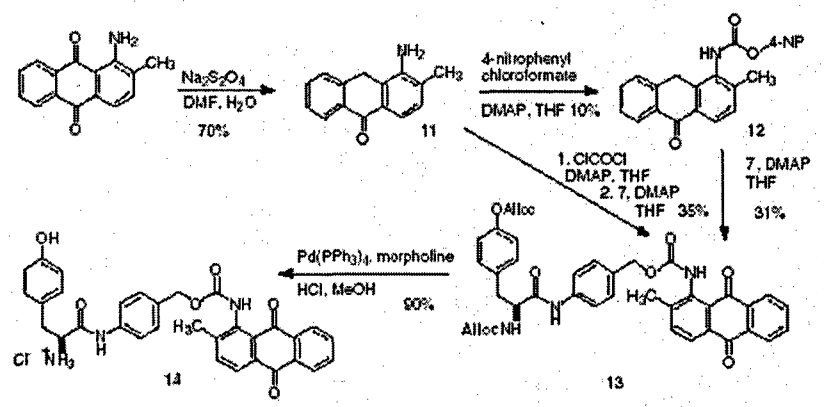
Scheme 3. Initial route to tyrosyl rhodamine conjugate via *p*-aminobenzyl carbamate linker.



Scheme 4. Y-alloc route to tyrosyl rhodamine conjugate.



Scheme 5. Preparation of tyrosyl aminomethylcoumarin conjugate.



Scheme 6. Preparation of tyrosyl aminomethylanthraquinone conjugate.

of the hydrochloride salt of anthraquinone substrate **14** in good yield.

With three substrates in hand, spectroscopic and enzymatic studies were conducted to establish proof of concept for use as image contrast agents. Enzymatic release of the fluorophores was initially probed using fresh, enzymatically active PSA and chymotrypsin, using UV detection to quantitate (and fluorescence in the case of **10**). Release of fluorophore correlated with release of *p*-aminobenzyl alcohol and tyrosine, confirming the function of the self-immolative linker. As can be seen (Table 1), though proof-of-concept is established, in the present examples, chymotrypsin is more effective than PSA at cleavage. Though this is unsurprising, more complex oligopeptide substrates are known whose specificity for PSA outranks chymotrypsin significantly, the most selective of these (HSSKLQ), which will now become the target of future synthetic studies and kinetic analysis.[8] Additionally, linker architecture has been shown to have a marked impact on substrate half-life in three-component systems,[9,12] suggesting that specificity and stability might ultimately be tailored according to desired application. Though the

Table 1. Enzyme mediated release of chromophores[a]

Entry	Substrate	UV λ_{max} conjugate	UV λ_{max} free	Chymotrypsin[b]	PSA[b]
1	**6**	300	497	9	4
2	**10**	328[c]	352[d]	24	10
3	**14**	285	486	18	6

[a] In duplicate. PSA (Cortex Biochem), 20 μL, 0.5 mg/mL, pH 7.4 0.01 M phosphate buffer saline [PBS]) or α-chymotrypsin (Sigma, TLCK-treated, 20 μL, 0.5 mg/mL, pH 7.4 0.01 M PBS), 160 μL PBS, and 20 μL substrate (1.0 mg/mL; 1:1, EtOH:H_2O) were incubated for 24 h at 37 °C. Duplicate control reactions containing PBS (180 μL) and substrate (20 μL) solutions were incubated under identical conditions. *p*-Aminobenzyl alcohol was quantified against authentic standards by HPLC (C18 μBondpak, 1 mL/min, 100% *i*PrOH, t_R = 8.2 min). Specific activity was determined on the basis of mM released fluorophore per unit time per unit mass of enzyme.

[b] mM/h/mg fluorophore released.

[c] Fluorescence emission λ_{max} 397.

[d] Fluorescence emission λ_{max} 435.

changes in the λ_{max} range between free and bound contrast agents prepared are pronounced, differences in fluorescent characteristics will be of more importance for imaging purposes where differences in quantum yield might be exploited.[13]

Moreover, for application with in vitro and in vivo analysis it will be necessary to employ fluorophores with spectral characteristics tailored to match imaging devices. Contrast agents in the near IR range (e.g., the Cy dye family) may prove desirable,[14] in that the conjugated amino function has a profound influence on its quantum yield.[15] The coupling chemistries described herein for amino substituted fluorophores offer flexibility towards this goal, providing the potential for in situ CCD based near-IR imaging of systemic agents that are locally activated under in vivo conditions.[14]

In summary, a three-component system comprised of enzyme substrate, inert linker and fluorophore has been designed and activation by chymotrypsin and PSA demonstrated. The results support the synthesis and in vitro evaluation of more complex and selective substrates, which will be reported in due course.

Acknowledgements

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References and notes

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